CORN ROOT PREFERENTIAL PROMOTERS AND USES THEREOF.

[001] FIELD OF THE INVENTION

[002] The invention relates to the isolation of promoters from corn capable of directing transcription of an operably linked foreign DNA sequence preferentially, selectively or exclusively in the roots of plants, such as corn plants. The invention also relates to the use of chimeric genes for the preferential or selective expression of biologically active RNA of interest in the roots of plants, such as corn plants. Plants, such as corn plants, comprising corn root preferential or selective promoters operably linked to a foreign DNA sequence which, upon transcription, yield biologically active RNA preferentially or selectively in the roots of plants are also provided.

[003] DESCRIPTION OF RELATED ART

[004] A significant consideration in the production of transgenic plants is obtaining sufficient levels of expression of the transgene in the tissues of interest in a preferential or selective way. In this way, potential drawbacks associated with the constitutive expression of the transcript, such as yield drag, may be avoided. Selection of appropriate promoters is crucial for obtaining the pattern of expression of interest with a particular transgene.

[005] Selective expression of transgenes in roots of plants, particularly cereal plants, such as corn, is considered to be potentially commercially important, e.g. for alteration of the function of root tissue, resistance to pathogens or pests with a preference for attack of roots (such as nematodes, corn rootworm etc.), resistance to herbicides or adverse environmental conditions (such as drought or soil composition).

[006] US 5,633,363 describes a 4.7 kb upstream promoter region designated ZRP2 isolated from maize and attributes a particular utility to this promoter region in driving root preferential expression of heterologous genes.

[007] WO 97/44448 relates generally to mechanisms of gene expression in plants and more specifically to regulation of expression of genes in plants in a tissue-specific manner particularly in roots. A method for isolation of transcriptional regulatory elements that contribute to tissue-preferred gene expression is disclosed.

[008] WO 00/15662 describes a promoter of a glycine rich protein (zmGRP3) whose transcripts accumulate exclusively in roots of young maize seedlings following developmentally specific patterns.

[009] WO 00/070068 and WO 00/70066 describe respectively the maize RS81 and RS324 promoters which are promoters of genes expressed in maize root tissue but not in kernel tissue and in molecular analysis were described to have a root-specific expression profile.

[010] Despite the fact that corn root preferential promoters are available in the art, a need remains for alternative promoters capable of preferential or selective root selective expression, e.g. for the independent expression of several foreign DNA sequences of interest without the possibility of post-transcriptional silencing due to the use of the same promoter. In addition, the known corn root preferential promoters, each direct a particular temporal, spatial and/or developmental expression pattern, which does not always suit particular goals. There remains thus a need for novel corn root preferential promoters with the capacity to control transcription in roots, preferably in a more selective manner, and also preferably resulting in a highly abundant transcription product.

[011] SUMMARY AND OBJECTS OF THE INVENTION

[012] It is an object of the invention to provide corn root preferential promoters comprising a nucleotide sequence selected from the following group of nucleotide sequences:

a. a nucleotide sequence comprising the nucleotide sequence of SEQ ID No 1 from the nucleotide at position 1 to the nucleotide at position 338 or the nucleotide sequence of SEQ ID No 2 from the nucleotide sequence at position 11 to the nucleotide at position 1196 ("GL4 promoter";

b. a nucleotide sequence comprising the nucleotide sequence of SEQ ID No 14 from the nucleotide at position 1 to the nucleotide at position 1280 ("GL5 promoter");

c. a nucleotide sequence comprising the nucleotide sequence of about an 400 bp to an about 1300 bp DNA fragment from the 5' end of a corn root preferential gene encoding a mRNA, from which a cDNA can be prepared that comprises the complement of the nucleotide sequence of SEQ ID No 7 or SEQ ID No 8 or SEQ ID No 9 or SEQ ID No 10;

d. a nucleotide sequence comprising the nucleotide sequence of an about 400 bp to an about 1300 bp DNA fragment from the 5' end of a corn root preferential gene encoding a mRNA from which a cDNA can be prepared that contains a nucleotide sequence encoding a polypeptide with the amino acid of SEQ ID No 4 or 6;

e. a nucleotide sequence comprising the nucleotide sequence of an about 400 bp to an about 1300 bp DNA fragment from the 5' end of a corn root preferential gene encoding a mRNA, from which a cDNA can be prepared that comprises a nucleotide sequence having at least 75

%, at least 80 %, at least 90%, at least 95%, or is identical to the nucleotide sequence of any of SEQ ID No 3, 5 or 11;

f. a nucleotide sequence comprising the nucleotide sequence having at least 70% at least 80 %, at least 90%, at least 95%, or is identical to any of said nucleotide sequence mentioned under a), b), c),d), e), or f) ; or

g. a nucleotide sequence comprising the nucleotide sequence of an about 400 bp to an about 1300 bp DNA fragment hybridizing under stringent conditions with a DNA fragment having said nucleotide sequence mentioned under a), b), c), d), e) or f).

[013] The corn root preferential promoters may be comprised within a corn root preferential promoter region, and may further comprise the nucleotide sequence of SEQ ID 1 from the nucleotide at position 339 to the nucleotide at position 366 or the nucleotide sequence of SEQ ID 14 from the nucleotide at position 1281 to the nucleotide at position 1308.

[014] It is another object of the invention to provide chimeric genes comprising the following operably linked DNA regions: a corn root preferential promoter according to the invention; a heterologous DNA region encoding a biologically active RNA of interest; and a transcription termination and polyadenylation signal active in plant cells. The biologically active RNA may encode a protein of interest, such as a protein which when expressed in the cells of a plant confers pest or pathogen resistance to said plant. The biologically active RNA may also be an antisense, sense or double stranded RNA useful for post-transcriptional silencing of a target gene of interest.

[015] Also provided are plant cells and plants or seeds thereof, particularly cereal plants, such as corn plants comprising a chimeric gene according to the invention.

[016] It is yet another objective to provide a method for expressing a biologically active RNA preferentially in the roots of a plant, such as a corn plant, comprising the

steps of: providing the cells of the roots of said plants with a chimeric gene according to the invention; and growing the plants.

[017] The invention further provides the use of a corn root preferential promoter according to the invention for preferential expression of a biologically active RNA in roots of a plant, such as a corn plant.

[018] It is yet another object of the invention to provide isolated DNA molecules comprising a nucleotide sequence encoding a protein comprising the amino acid sequence of SEQ ID No 4 or SEQ ID No 6, particularly a nucleotide sequence selected from the group of SEQ ID No 3; SEQ ID No 5 and SEQ ID No 11 and the use thereof for isolation of a corn root preferential promoter or promoter region.

[019] In yet another embodiment the invention provides a method for isolating a corn root preferential promoter region, comprising the steps of: identifying a genomic fragment encoding an RNA transcript from which a cDNA can be synthesized, which cDNA comprises the nucleotide sequence of SEQ ID 3 or SEQ ID No 5 or functional equivalents; and isolating a DNA region upstream of a nucleotide sequence encoding the protein with the amino acid of SEQ ID No 4 or SEQ ID No 6 or functional equivalents. Also provided are corn root preferential promoters obtained by this method.

[020] BRIEF DESCRIPTION OF THE FIGURES.

[021] Figure 1: Alignment of the nucleotide sequences for cDNAs GL4, GL5 and GL12. Gaps in the sequence introduced for optimal alignment are indicated by a dash.

[022] Figure 2: Nucleotide sequence for the short corn root preferential promoter region from GL4.

[023] Figure 3: Nucleotide sequence for the long corn root preferential promoter region from GL4.

[024] Figure 4: Nucleotide sequence for the corn root preferential promoter region from GL5.

[025] Figure 5: Schematic representation of pTWV011. LB: left T-DNA border; 3'nos: 3' end of the nopaline synthase gene; bar: bialaphos resistance coding region; P35S3: promoter region of the 35S transcript of CaMV; 3' 35S: 3' end of the 35S transcript of CaMV; isp1a: coding region for insecticidal secreted protein 1a from *Brevibacillus laterosporus*; 5'gl4: leader region of the GL4 promoter region; Pgl4: corn root selective promoter GL4; isp2a: coding region for insecticidal secreted protein 2a from *Brevibacillus laterosporus*; RB: right T-DNA border region; nptI homology: region of homology with helper Ti-plasmids; ORI colE1: colE1 origin of replication; ORI pVS1: origin of replication for *Pseudomonas*; PaadA: bacterial promoter of the aminoglycoside adenyltransferase conferring resistance to streptomycin and spectinomycin; aadA: coding region of the aminoglycoside adenyltransferase gene; 3' aadA: 3' end of the aminoglycoside adenyltransferase gene.

[026] Figure 6: Schematic representation of pTWV018. LB: left T-DNA border; 3'nos: 3' end of the nopaline synthase gene; bar: bialaphos resistance coding region; P35S3: promoter region of the 35S transcript of CaMV; 3' 35S: 3' end of the 35S transcript of CaMV; isp1a: coding region for insecticidal secreted protein 1a from *Brevibacillus laterosporus*; 5'gl5: leader region of the GL5 promoter region; Pgl5: corn root selective promoter GL5; isp2a: coding region for insecticidal secreted protein 2a from *Brevibacillus laterosporus*; RB: right T-DNA border region; nptI homology: region of homology with helper Ti-plasmids; ORI colE1: colE1 origin of replication; ORI pVS1: origin of replication for *Pseudomonas*; PaadA: bacterial promoter of the aminoglycoside adenyltransferase conferring resistance to streptomycin and spectinomycin; aadA: coding region of the aminoglycoside adenyltransferase gene; 3' aadA: 3' end of the aminoglycoside adenyltransferase gene.

[027] DETAILED DESCRIPTION OF EMBODIMENTS OF THE INVENTION

[028] The invention is based on the finding that the promoters described herein are particularly suited for the preferential and abundant expression (*i.e.* transcription or transcription and translation) of an operably linked foreign DNA in roots of plants, particularly cereal plants such as corn.

[029] In one embodiment of the invention, a corn root preferential promoter region is provided comprising the nucleotide sequence of SEQ ID 1 of about 400 bp. In another embodiment, a corn root preferential promoter region is provided comprising the nucleotide sequence of SEQ ID No 2 of about 1200 bp. In yet another embodiment, a corn root preferential promoter region is provided comprising the nucleotide sequence of SEQ ID No 14 from the nucleotide at position 1 to the nucleotide at position 1280.

[030] As used herein "corn" refers to maize i.e. *Zea mays L.*

[031] As used herein, the term "promoter" denotes any DNA which is recognized and bound (directly or indirectly) by a DNA-dependent RNA-polymerase during initiation of transcription. A promoter includes the transcription initiation site, and binding sites for transcription initiation factors and RNA polymerase, and can comprise various other sites (*e.g.,* enhancers), at which gene expression regulatory proteins may bind.

[032] The term "regulatory region", as used herein, means any DNA, that is involved in driving transcription and controlling (*i.e.,* regulating) the timing and level of transcription of a given DNA sequence, such as a DNA coding for a protein or polypeptide. For example, a 5' regulatory region (or "promoter region") is a DNA sequence located upstream (*i.e.,* 5') of a coding sequence and which comprises the promoter and the 5'-untranslated leader sequence. A 3' regulatory region is a DNA sequence located downstream (*i.e.,* 3') of the coding sequence and which comprises suitable transcription 3' end formation (and/or regulation) signals, including one or more polyadenylation signals.

[033] The term "gene" means any DNA fragment comprising a DNA region (the "transcribed DNA region") that is transcribed into a RNA molecule (*e.g.,* a mRNA) in a cell under control of suitable regulatory regions, e.g., a plant expressible promoter region. A gene may thus comprise several operably linked DNA fragments such as a promoter, a 5' untranslated leader sequence, a coding region, and a 3' untranslated region comprising a polyadenylation site. An endogenous plant gene is a gene which is naturally found in a plant species. A chimeric gene is any gene which is not normally found in a plant species or, alternatively, any gene in which the promoter is not associated in nature with part or all of the transcribed DNA region or with at least one other regulatory regions of the gene.

[034] The term "expression of a gene" refers to the process wherein a DNA region under control of regulatory regions, particularly the promoter, is transcribed into an RNA which is biologically active, *i.e.,* which is either capable of interaction with another nucleic acid or which is capable of being translated into a biologically active polypeptide or protein. A gene is said to encode an RNA when the end product of the expression of the gene is biologically active RNA, such as an antisense RNA or a ribozyme. A gene is said to encode a protein when the end product of the expression of the gene is a biologically active protein or polypeptide.

[035] The term "root-selective", with respect to the expression of a DNA in accordance with this invention, refers to, for practical purposes, the highly specific, expression of a DNA in cells of roots of plants, such as corn plants ("corn-root-selective"). In other words, transcript levels of a DNA in tissues different of root plants is either below detection or very low (less than about 0.2 picograms per microgram total RNA).

[036] The term "root-preferential" with respect to the expression of a DNA in accordance with this invention, refers to an expression pattern whereby the DNA is expressed predominantly in roots, but expression can be identified in other tissues of

the plant. In one embodiment of the present invention, expression in roots may be enhanced by about 2 to about 10 times higher in roots than in other tissues.

[037] It will be clear that having read these embodiments, the person skilled in the art can easily identify and use functional equivalent promoters for the same purposes.

[038] DNA sequences which have a promoter activity substantially similar to the corn root preferential promoters comprising the nucleotide sequence of SEQ ID 1 from the nucleotide at position 1 to the nucleotide at position 338 or SEQ ID 2 from the nucleotide at position 11 to the nucleotide at position 1196 or SEQ ID 14 from the nucleotide at position 1 to the nucleotide at position 1280, or parts thereof having promoter activity, are functional equivalents of these promoters. These functional equivalent promoters may hybridize with the corn root preferential promoter regions comprising the nucleotide sequence of SEQ ID 1 or of SEQ ID No 2 or of SEQ ID 14 under stringent hybridization conditions.

[039] "Stringent hybridization conditions" as used herein means that hybridization will generally occur if there is at least 95% or even at least 97% sequence identity between the probe and the target sequence. Examples of stringent hybridization conditions are overnight incubation in a solution comprising 50% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 µg/ml denatured, sheared carrier DNA such as salmon sperm DNA, followed by washing the hybridization support in 0.1 x SSC at approximately 65 °C. Other hybridization and wash conditions are well known and are exemplified in Sambrook et al, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY (1989), particularly chapter 11.

[040] Other functional equivalent promoters comprise nucleotide sequences which can be amplified using oligonucleotide primers comprising at least about 25, at least about 50, or at least about 100 consecutive nucleotides selected from the nucleotide sequence of SEQ ID 1 or SEQ ID 2, in a polymerase chain amplification reaction.

Examples of such oligonucleotide primers are GVK 29 (SEQ ID No 9) and GVK 30 (SEQ ID No 10).

[041] Functionally equivalent promoters may be isolated e.g. from different corn varieties. They may also be made by modifying isolated corn root-preferential promoters through addition, substitution, deletion or insertion of nucleotides. They can also be completely or partly synthesized.

[042] Alternatively, functional equivalent promoters may be isolated using a cDNA of a transcript which is expressed at a high level in roots of a plant, such as a corn plant, as a probe to isolate the genomic DNA upstream of the nucleotide sequence corresponding to the nucleotide sequence of the cDNA. As used herein "cDNA" is used to indicate both the first strand cDNA (complementary to the mRNA) as well as the strand complementary thereto (and thus identical to the mRNA except that U is replaced by T) or a double stranded cDNA fragment. In accordance with this invention, corn root selective cDNAs and their corresponding plant genomic DNA fragments may be identified as follows :

- a cDNA library may be constructed starting from mRNA isolated from roots and the cDNA library subjected to differential screening in order to identify an mRNA which is preferentially present in roots when compared to other plant tissues including but not limited to: leaves, seeds, stems, reproductive organs, and the like. Alternatively, the cDNA library may screened with oligonucleotides, that are deduced from a determined amino acid sequence of an isolated protein, that has been identified to be preferentially present in the roots. Furthermore, it is possible to use the same oligonucleotides in a nested-PCR approach and to use the amplified fragment(s) as a probe to screen the library. The corn root preferential cDNA library can be constructed from a pool of mRNAs, isolated at different stages of corn root development. One method to identify and isolate the 3' ends of cDNA of RNA particularly expressed in a specific tissue such as here the roots of plants, is the so-called READS analysis or Restriction-Enzyme digested cDNAs as described e.g. by

Prashar and Weismann or US patent 5712126 (both documents are herein incorporated by reference).

- A cDNA reverse transcribed from RNA preferentially transcribed in roots of plants, such as corn plants, or 3' ends of cDNAs identified by READS differential display analysis as expressed preferentially in roots of plants may be isolated and further characterized by e.g. nucleotide sequence determination; a full length cDNA may be isolated using e.g. 5' RACE (rapid amplification of cDNA ends) technology.
- This cDNA or the 3' end thereof may be used as a probe to identify and isolate the region in the plant genome, comprising the nucleotide sequence encoding the corn root preferential mRNA. Alternatively, the genomic DNA can be isolated by e.g. inverse PCR using oligonucleotides deduced from the cDNA sequence. Alternatively, TAIL-PCR (thermal assymetric interlaced PCR as described by Liu et al. (1995)) using nested long specific oligonucleotides derived from the nucleotide sequence of the (5' end) of the identified cDNA and a short arbitrary degenerate primer may be used to isolate the genomic sequences flanking the coding region.
- Optionally, RNA probes corresponding to the cDNAs are constructed and used in conventional RNA-RNA in-situ hybridization analysis [see e.g., De Block *et al.* (1993), *Anal. Biochem.* **215**: 86] of different plant tissues, including the root tissue of interest, to confirm the preferential presence of the mRNA produced by the endogenous plant gene presumed root preferential expression in those roots.

[043] Once the corn root-prefential gene (i.e., the genomic DNA fragment, encoding the corn root-preferential mRNA from which the corn-root preferential cDNA can be prepared) is obtained, the promoter region containing the corn root -preferential promoter is determined as the region upstream (i.e., located 5' of) from the codon coding for the first amino acid of the protein encoded by the mRNA. It is preferred that such promoter region is at least about 400 to 500 bp, at least about 1000 bp, about 1200 bp, about 1300 bp, or at least about 1500 to 2000 bp, upstream of the start codon. For convenience, such promoter region may not extend more than about

3000 to 5000 bp upstream of the start codon. The size fragment may be partially determined by the presence of convenient restriction sites. The actual corn root-preferential promoter is the region of the genomic DNA upstream (i.e., 5') of the region encoding the corn root-preferential mRNA. A chimeric gene comprising a corn root-preferential promoter operably linked to the coding region of a marker gene will produce the marker protein preferentially in the cells of the corn roots of transgenic corn plants, which can be assayed by conventional *in situ* histochemical techniques.

[044] Examples of corn root-preferential genes from which corn root-preferential promoters can be obtained, are genes, that encode a mRNA which can be detected preferentially in corn roots and have a size of about 600 nts, from which a cDNA can be prepared that contains the complement of the nucleotide sequence corresponding to the nucleotide sequence of oligonucleotide GVK27 (SEQ ID No 7) and/or the complement of the nucleotide sequence of oligonucleotide GVK28 (SEQ ID No 8); and/or contains the complement of the nucleotide sequence corresponding to the oligonucleotide GVK29 (SEQ ID No 9) and/or contains the complement of the nucleotide sequence corresponding to the oligonucleotide GVK30 (SEQ ID No 10) . Such corn root-prefential cDNA may contain each of the aforementioned sequences corresponding to oligonucleotides GVK27 and GVK28 as well as GVK29 or GVK30

[045] Such a gene is the gene that encodes a corn root-preferential transcript from which a cDNA can be prepared that contains a nucleotide sequence encoding the protein with the amino acid sequence of SEQ ID 4 and which may e.g. have the nucleotide sequence of SEQ ID No 3. Other corn root-preferential genes are the genes that encode a corn root-preferential mRNA from which a cDNA can be prepared that contains the sequence of SEQ ID No 5 or SEQ ID 11, or that contains a nucleotide sequence encoding the protein comprising the amino acid sequence of SEQ ID 6.

[046] One embodiment of a promoter of the present invention is a promoter contained in the 5' regulatory region of a genomic clone comprising a nucleotide

sequence corresponding to the cDNA with the nucleotide sequence of any one of SEQ ID No 5, 6 or 11, e.g. the 5' regulatory region with the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 11 to the nucleotide at position 1196 or a DNA fragment comprising the sequence of SEQ ID No 2 starting anywhere between the nucleotide at position 11 to the nucleotide at postion 859, and ending at nucleotide position 1233 (just before the ATG translation start codon) or a DNA fragment comprising the sequence of SEQ ID No 14 from the nucleotide at position 1 to the nucleotide at position 1280. Such a promoter region comprises a corn root-preferential promoter of the invention and the 5' untranslated leader region, and may be used for the construction of root-preferential chimeric genes, particularly corn root preferential chimeric genes. However, smaller DNA fragments can be used as promoter regions in this invention and it is believed that any fragment of the DNA of SEQ ID No 2 which comprises at least the about 400 basepairs upstream from the translation inititation codon can be used.

[047] Artificial promoters can be constructed which contain those internal portions of the promoter of the 5' regulatory region of SEQ ID No 1 or SEQ ID No 2 or SEQ ID 14 that determine the corn root-preference of this promoter. These artifical promoters might contain a "core promoter" or "TATA box region" of another promoter capable of expression in plants, such as a CaMV 35S "TATA box region" as described in W0 93/19188. The suitability of promoter regions containing such artificial promoters may be identified by their appropriate fusion to a reporter gene and the detection of the expression of the reporter gene in the appropriate tissue(s) and at the appropriate developmental stage. It is believed that such smaller promoters and/or artificial promoters comprising those internal portions of the 5' regulatory region of SEQ ID No. 1 or 2 that determine the corn root preference can provide better selectivity of transcription in corn root cells and/or provide enhanced levels of transcription of the transcribed regions of the corn root-preerential chimeric genes of the invention. Such smaller portions of the corn root preferential promoter regions of the invention may

include the nucleotide sequences which share a high homology between the GL4 and GL5 promoter regions such as: the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 1024 to the nucleotide at position 1105 (having a 80 % match with the nucleotide sequence of SEQ ID No 14 from the nucleotide at position 435 to the nucleotide at position 510); the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 866 to the nucleotide at position 994 (having a 77 % match with the nucleotide sequence of SEQ ID No 14 from the nucleotide at position 236 to the nucleotide at position 358); the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 544 to the nucleotide at position 568 (having a 96% match with the nucleotide sequence of SEQ ID No 15 from the nucleotide at position 198 to the nucleotide at position 222); the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 1122 to the nucleotide at position 1143 (having a 73 % match with the nucleotide sequence of SEQ ID No 15 from the nucleotide at position 485 to the nucleotide at position 510).

[048] Besides the actual promoter, the 5' regulatory region of the corn root-preferential genes of this invention also comprises a DNA fragment encoding a 5' untranslated leader (5'UTL) sequence of an RNA located between the transcription start site and the translation start site. It is believed that the 5' transcription start site of the GL4 promoter is located around position 1197 in SEQ ID No 2, resulting in a 5'UTL of about 30 nucleotides in length. It is believed that the 5' transcription start site of the GL5 promoter is located around position 1280 in SEQ ID No 14, resulting in a 5'UTL of about 30 nucleotides in length It is also believed that this region can be replaced by another 5'UTL, such as the 5'UTL of another plant-expressible gene, without substantially affecting the specificity of the promoter.

[049] Thus, in another embodiment the invention provides a corn root preferential promoter or corn root preferential promoter region comprising a nucleotide sequence selected from the following group of nucleotide sequences:

- a nucleotide sequence comprising the nucleotide sequence of SEQ ID No 1 from the nucleotide at position 1 to the nucleotide at position 338 or the nucleotide sequence of SEQ ID No 2 from the nucleotide sequence at position 11 to the nucleotide at position 1196;
- a nucleotide sequence comprising the nucleotide sequence of SEQ ID No 14 from the nucleotide at position 1 to the nucleotide at position 1280;
- the nucleotide sequence of about an 400 bp to an about 1300 bp DNA fragment from (the 5' end of) a corn root preferential gene encoding a mRNA, the mRNA having a size of about 600 nt, from which a cDNA can be prepared that contains the complement of the nucleotide sequence corresponding to the nucleotide sequence of SEQ ID No 7, SEQ ID No 8, SEQ ID No 9, or SEQ ID No 10;
- the nucleotide sequence of an about 400 bp to an about 1300 bp DNA fragment from the 5' end of a corn root prefential gene encoding a mRNA, the mRNA having a size of about 600 nt, from which a cDNA can be prepared that contains a nucleotide sequence encoding a polypeptide with the amino acid of SEQ ID No 4 or 6;
- the nucleotide sequence of an about 400 bp to an about 1300 bp DNA fragment from the 5' end of a corn root preferential gene encoding a mRNA, from which a cDNA can be prepared that comprises a nucleotide sequence having at least 75 % or at least 80 % or at least 90%, or at least 95% sequence identity with the nucleotide sequence of any of SEQ ID No 3, 5 or 11 or is identical thereto;
- the nucleotide sequence having at least 70% or 80 % or 90% or 95% sequence identity to any of the nucleotide sequence mentioned under a), b), c),d), e), or f), particularly the nucleotide sequence mentioned under a) or is identical thereto; or
- the nucleotide sequence of an about 400 bp to an about 1300 bp DNA fragment hybridizing under stringent conditions with a DNA fragment having the

nucleotide sequence mentioned under a), b), c), d), e) or f), particularly the nucleotide sequence mentioned under a).

[050] For the purpose of this invention, the "sequence identity" of two related nucleotide or amino acid sequences, expressed as a percentage, refers to the number of positions in the two optimally aligned sequences which have identical residues (x100) divided by the number of positions compared. A gap, *i.e.* a position in an alignment where a residue is present in one sequence but not in the other, is regarded as a position with non-identical residues. The alignment of the two sequences is performed by the Needleman and Wunsch algorithm (Needleman and Wunsch 1970). The computer-assisted sequence alignment above can be conveniently performed using standard software program such as GAP which is part of the Wisconsin Package Version 10.1 (Genetics Computer Group, Madison, Wisconsin, USA) using the default scoring matrix with a gap creation penalty of 50 and a gap extension penalty of 3.

[051] Promoters and promoter regions of the invention may also comprise additional elements known to improve transcription efficiency such as enhancers, introns, etc.

[052] The invention further includes DNA molecules comprising the corn root preferential promoters of the invention operably linked to one or more heterologous regions coding for a biologically active RNA, peptide or protein. The promoters of the invention may be used to express any heterologous coding region desired.

[053] Thus in another embodiment of the invention, a chimeric gene is provided comprising

a. a corn root preferential promoter region; comprising the nucleotide sequence selected from the group consisting of
    i. the nucleotide sequence of SEQ ID No 1 from the nucleotide at position 1 to the nucleotide at position 338 or the nucleotide

sequence of SEQ ID No 2 from the nucleotide at position 11 to the nucleotide at position 1196;

ii. a nucleotide sequence comprising the nucleotide sequence of SEQ ID No 14 from the nucleotide at position 1 to the nucleotide at position 1280;

iii. the nucleotide sequence of about an 400 bp to an about 1300 bp DNA fragment from (the 5' end of) a corn root preferential gene encoding a mRNA, the mRNA having a size of about 600 nt, from which a cDNA can be prepared that contains the complement of the nucleotide sequence corresponding to the nucleotide sequence of SEQ ID No 7 SEQ ID No 8 or SEQ ID No 9 or SEQ ID No 10;

iv. the nucleotide sequence of an about 400 bp to an about 1300 bp DNA fragment from the 5' end of a corn root preferential gene encoding a mRNA, the mRNA having a size of about 600 nt, from which a cDNA can be prepared that contains a nucleotide sequence encoding a polypeptide with the amino acid of SEQ ID No 4 or 6;

v. the nucleotide sequence of an about 400 bp to an about 1300 bp DNA fragment from the 5' end of a corn root preferential gene encoding a mRNA, from which a cDNA can be prepared that comprises a nucleotide sequence having at least 75 % or 80% or 90% or 95% sequence identity with the nucleotide sequence of any of SEQ ID No 3, 5 or 11 or is identical thereto;

vi. the nucleotide sequence having at least 75 % or 80% or 90% or 95% sequence identity with the nucleotide sequence mentioned under i), ii), iii),iv), v), or vi), particularly the

nucleotide sequence mentioned under i) or is identical thereto; or

vii. the nucleotide sequence of an about 400 bp to an about 1300 bp DNA fragment hybridizing under stringent conditions with a DNA fragment having the nucleotide sequence mentioned under i), ii), iii), iv), v) or vi), particularly the nucleotide sequence mentioned under a);

b. a DNA region of interest, which when transcribed yields a biologically active RNA; and

c. a DNA region comprising a 3' transcription termination and polyadenylation signal functional in plant cells.

[054] The DNA region of interest, or the transcribed RNA may thus encode a protein or polypeptide, but may also encode biologically active RNA, such as an antisense RNA, a sense RNA, or a dsRNA comprising both sense and antisense RNA stretches capable of basepairing and forming a double stranded RNA, as described in WO 99/53050 (incorporated herein by reference) usable for posttranscriptional gene silencing of a target sequence.

[055] To confer corn rootworm resistance, such as for example resistance to *Diabrotica barberi, Diabrotica undecimpuncata,* and/or *Diabrotica virgifera,* to plants, such as corn plants, in a root selective or root preferential way, suitable candidate DNA regions to be operably linked to the corn root selective promoters of the invention include the mature VIP1Aa protein when combined with the mature VIP2Aa or VIP2Ab protein of PCT publication WO 96/10083; the corn rootworm toxins of *Photorhabdus* or *Xhenorhabdus* spp., e.g., the insecticidal proteins of *Photorhabdus luminescens* W-14 (Guo et al., 1999, J. Biol. Chem. 274, 9836-9842); the CryET70 protein of WO 00/26378; the insecticidal proteins produced by Bt strains PS80JJ1, PS149B1 and PS167H2 as described in WO 97/40162, particularly the about 14 kD and about 44 kD proteins of Bt strain PS149B1; the Cry3Bb protein of US patent

6,023,013; protease inhibitors such as the N2 and R1 cysteine proteinase inhibitors of soybean (Zhao et al., 1996, Plant Physiol. 111, 1299-1306) or oryzastatine such as rice cystatin (Genbank entry S49967), corn cystatin (Genbank entries D38130, D10622, D63342) such as the corn cystatin expressed in plants as described by Irie et al. (1996, Plant Mol. Biol. 30, 149-157). Also included are all equivalents and variants, such as truncated proteins retaining insecticidal activity, of any of the above proteins.

[056] In one embodiment of the invention, chimeric genes for conferring rootworm resistance in a root preferential way comprise a nucleotide sequence encoding an insecticidal secreted protein (ISP) from *Brevibacillus laterosporus,* which is insecticidal when ingested by an insect in combination with an ISP complimentary protein, such as another ISP protein, as described in PCT application PCT/EP01/05702 published as WO 01/87931 (incorporated herein by reference; particularly DNA sequences SEQ ID No7 and 9 of WO 01/87931). The nucleotide sequences encoding ISP protein may be a modified DNA.

[057] The invention further provides methods for expressing a foreign DNA of interest preferentially in the roots of a plant, such as a corn plant, comprising the following steps:

providing plant cells with the chimeric genes of the invention, which may be stably integrated in their genome, e.g. their nuclear genome, to generate transgenic cells; and regenerating plants from said transgenic cells.

[058] A convenient way to provide plant cells with the chimeric genes of the invention is to introduce the DNA via conventional transformation methods. It will be clear that actual method of transforming the plants, particularly cereal plants has little importance for the currently described methods. Several methods for introduction of foreign DNA into the genome of plant cells are available in the art. These methods include, but are not limited to, direct protoplast transformation (see e.g. for corn US 5792936, incorporated herein by reference); *Agrobacterium*-mediated transformation

(see e.g. for corn US 6074877 or US 6140553 incorporated herein by reference); microprojectile bombardment, electroporation of compact embryogenic calli (see e.g. for corn US 5641664 incorporated herein by reference); or silicon whisker mediated DNA introduction.

[059] Operably linking the foreign DNA of interest to a corn root preferential promoter according to the invention may also be achieved by replacing the DNA naturally associated with the corn root preferential promoter by homologous recombination with the DNA of interest, provided that said DNA of interest comprises a homology region with the DNA normally associated with the corn root preferential promoter. Methods for introducing DNA of interest into plant cell genome by homologous recombination are available (e.g. US patent 5744336 incorporated herein by reference).

[060] The obtained transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the chimeric gene for corn root preferential expression according to the invention in other varieties of the same or related plant species, or in hybrid plants. Seeds obtained from the transformed plants contain the chimeric genes of the invention as a stable genomic insert and are also encompassed by the invention.

[061] It will be appreciated that the means and methods of the invention are particularly useful for corn, but may also be used in other plants with similar effects, particularly in cereal plants including corn, wheat, oat, barley, rye, rice, turfgrass, sorghum, millet or sugarcane plants.

[062] The following non-limiting Examples describe the isolation of corn root preferential promoters, and the construction of chimeric genes for selective expression in corn root plants. Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, NY and in Volumes 1 and 2 of

Ausubel *et al.* (1994) *Current Protocols in Molecular Biology, Current Protocols*, USA. Standard materials and methods for plant molecular work are described in *Plant Molecular Biology Labfax* (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK.

[063] Throughout the description and Examples, reference is made to the following sequences represented in the sequence listing.

SEQ ID No 1: nucleotide sequence of the about 400 bp corn root preferential promoter (GL4 promoter).

SEQ ID No 2: nucleotide sequence of the about 1200 bp corn root preferential promoter (GL4 promoter).

SEQ ID No 3: nucleotide sequence of the cDNA of the naturally associated mRNA transcribed under control of the corn root preferential promoter having the nucleotide sequence of SEQ ID No 1 (GL4).

SEQ ID No 4: amino acid sequence of the protein encoded by the cDNA GL4.

SEQ ID No 5: nucleotide sequence of the corn root preferential cDNA GL5.

SEQ ID No 6: amino acid sequence of the protein encoded by the cDNA GL5.

SEQ ID No 7: oligonucleotide primer GVK 27.

SEQ ID No 8: oligonucleotide primer GVK28.

SEQ ID No 9: oligonucleotide primer GVK 29.

SEQ ID No 10: oligonucleotide primer GVK 30.

SEQ ID No 11: nucleotide sequence of corn root preferential cDNA GL12.

SEQ ID No 12: nucleotide sequence of plasmid pTWV011.

SEQ ID No 13: nucleotide sequence of plasmid pTWV018.

SEQ ID No 14: nucleotide sequence of the about 1300 bp corn root preferential promoter (GL5 promoter).

SEQ ID No 15: oligonucleotide primer GVK22.

SEQ ID No 16: oligonucleotide primer GVK23.

SEQ ID No 17: oligonucleotide primer GVK24.

SEQ ID No 18: oligonucleotide primer GVK25.

SEQ ID No 19: oligonucleotide primer GVK26.

SEQ ID No 20: oligonucleotide primer GVK31.

SEQ ID No 21: oligonucleotide primer GVK32.

SEQ ID No 22: oligonucleotide primer GVK33.

SEQ ID No 23: oligonucleotide primer GVK38.

SEQ ID No 24: oligonucleotide primer GVK39.

SEQ ID No 25: oligonucleotide primer GVK45.

SEQ ID No 26: oligonucleotide primer MDB285.

SEQ ID No 27: oligonucleotide primer MDB286.

SEQ ID No 28: oligonucleotide primer MDB363.

SEQ ID No 29: oligonucleotide primer MDB364.

SEQ ID No 30: oligonucleotide primer MDB552.

SEQ ID No 31: oligonucleotide primer MDB556.

[064] EXAMPLES

[065] *Example 1. Isolation of root preferential corn cDNAs*

[066] RNA transcript tags which are expressed preferentially in maize roots were identified by a differential RNA cDNA display known as READS (Prashar and Weismann, 1999, *Methods in Enzymology* **303**:258- 272).

[067] To this end, total RNA samples were prepared from different tissues (roots, stems, leaves, ..) of corn plants, harvested at different developmental stages (from 64 day old plants to adult plants). 3' end fragments of cDNA molecules digested by different endorestriction nucleases were amplified using stringent PCR conditions and oligonucleotides as described by Prashar and Weismann (1999, supra) for each of the samples.

[068] Comparison of gel patterns of the 3' end cDNA restriction fragments generated for each of the RNA samples allowed a preliminary identification of fragments which appeared only in the corn root tissue RNA sample or were more prominent in root tissue RNA than in other corn tissue. These 3' end fragments were isolated and sequenced. Their nucleotide sequence was compared against public and proprietary databases and only novel sequences were used in a further Northern analysis using the RNA samples from different corn tissues as a driver and each of the isolated 3' end cDNA fragments as a probe. The hybridizing RNA transcripts were analyzed for size, abundance, and specificity. The results for the 3' end with the best specificity for expression in corn roots are summarized in Table 1, arranged in descending order of specificity and abundance.

[069] The 3' ends which hybridized to the most abundant RNA transcripts with the highest specific expression in corn roots (GL4; GL5 and GL12) were further analyzed.

[070] For GL4 and GL5 full length cDNAs were isolated using the SMART ^{TM} RACE cDNA amplification kit from CLONTECH Laboratories with nested oligonucleotide primers GVK22 (SEQ ID No 15)/GVK23 (SEQ ID No 16) for GL4 and GVK24 (SEQ

ID No 17)/GVK25 (SEQ ID No 18) for GL5 The nucleotide sequence of the full length cDNAs is represented in SEQ ID 3 and 5 respectively. Comparison of both nucleotide sequences revealed that GL4 and GL5 have about 89 % sequence identity.

[071] Table 1.

Identification of 3' end used as probe	Quantification of hybridizing transcript[1]	Estimated length of hybridizing transcript[2]	Specificity of presence of the hybridizing transcript
GL4	18	About 600	Root selective
GL5	15	About 600	Root selective
GL12	13	About 600	Root selective
GL11	3	About 820	Root selective
GL3	1	About 1200	Root selective
GL9	7	About 900	Root preferential
GL7	2	About 700	Root preferential
GL16	<2	About 1500	Low expression
GL17	<2	About 650	Low expression
GL6	---	---	No visible hybridization

[1] expressed in picogram/µg total RNA
[2] in nucleotide

[072] In both sequences, a small ORF could be identified (starting from the nucleotide of SEQ ID 3 at position 32 to the nucleotide at position 319 for GL4; the nucleotide of SEQ ID 5 at position 27 to the nucleotide at position 307 for GL5). The amino acid sequences of the polypeptides encoded by the ORFs are represented in SEQ ID No 4 or 6. The nucleotide sequence in the region encoding the ORF is more conserved between GL4 and GL5 cDNA than elsewhere in the fragments.

[073] Using the GL4 cDNA nucleotide sequence as a query, a nucleotide sequence has been identified with 91% sequence identity in the 322-nucleotide overlap (SEQ ID No 19 from WO 00/32799). It has not been described that SEQ ID No 19 from *Zea mays* as described in WO 00/32799 is transcribed in a root selective or root preferential way. Further, a nucleotide sequence (clone MEST23-CO7.T3 from a

seedling and silk cDNA library) has been identified having 99% sequence identity over 582 nt.

[074] Using the GL5 cDNA nucleotide sequence as a query, a nucleotide sequence has been identified with 85% sequence identity with FtsZ1 related sequence from *Zea mays* (A47325 in Geneseq). It has not been described that this sequence is transcribed in a root selective or root preferential way. Further, a nucleotide sequence (clone MEST523-G12 (3') from a seedling and silk cDNA library) has been identified having 100% sequence identity over 525 nt.

[075] *Example 2. Isolation of corn root preferential promoter regions of the gene transcribing a mRNA, the cDNA of which corresponds to GL4 or GL5 cDNA.*

[076] The genomic fragments upstream of the nucleotide sequences corresponding to GL4 cDNA and GL5 cDNA sequences, comprising the promoter region were isolated using Thermal Asymmetric Interlaced PCR as described by Liu et al (1995, *The Plant Journal* **8(3)**: 457-463)

[077] Corn genomic DNA for use as the initial template DNA was purified as described by Dellaporte et al. The sequence of the specific nested oligonucleotides used for TAIL-PCR to isolate the genomic fragments located upstream of the genomic DNA sequences corresponding to GL4 cDNA sequences are represented in SEQ ID No 7 (GVK27), SEQ ID No 8 (GVK28) and SEQ ID No 9 (GVK 29) ; the aspecific degenerate primers used were each of the 6 degenerate primers MDB285, MDB286, MDB363, MDB364, MDB552 or MDB556 in separate reactions. PCR conditions were as described in Liu et al (1995, supra).

[078] A genomic fragment of about 400 bp (corresponding to the amplification product obtained with the primer pair (GVK29/MDB285) was isolated, cloned in pGEM-T Easy ® and sequenced.

[079] Based on the nucleotide sequence of the about 400 bp fragment, new specific nested primer oligonucleotides (GVK 31/ SEQ ID No 20; GVK32 / SEQ ID No 21 and

GVK33 / SEQ ID No 22) were designed and used in conjunction with the above mentioned degenerated primers to isolate the adjacent DNA regions further upstream of the isolated promoter region fragment. This resulted in isolation of an about 350 nt DNA fragment (corresponding to the amplification product obtained with the primer pair GVK33 / MDB286) In a third round, the adjacent upstream DNA fragment of about 800 nt was isolated using new set of specific nested oligonucleotides GVK33 / SEQ ID No 22; GVK38 / SEQ ID No 23 and GVK39 / SEQ ID No 24 in conjunction with the above mentioned degenerated primers (corresponding to the amplification product using GVK39 and MDB363).

[080] To confirm the continuity of the isolated genomic upstream fragments, the complete 1200 bp DNA fragment was amplified using GVK29 and GVK45 (SEQ ID 25) primers and cloned in pGEM-T Easy®. The complete nucleotide sequence of the about 1200 bp upstream DNA fragment is represented in SEQ ID 2.

[081] Primers GVK 27, GVK28 and GVK30 (SEQ ID No 10) were used in conjunction with the above mentioned degenerated primers. An about 1300 nt fragment was amplified having the sequence of SEQ ID 14 (corresponding to the amplification product by MDB364 and GVK30).

[082] *Example 3. Construction of chimeric genes using the isolated GL4/GL5 corn root preferential promoter regions.*

[083] The following chimeric ISP1A/ISP2A constructs under the control of GL4 promoter region or under control of the GL5 promoter region were made using standard recombinant DNA methods:

[084] GL4::ISP1A comprising the following DNA fragments:

the GL4 promoter region (SEQ ID No 2);

a DNA fragment encoding the isp1A protein of *Brevibacillus laterosporus* (complement of the nucleotide sequence of SEQ ID 12 from the nucleotide at position 2003 to the nucleotide at position 4511);

the 3' end fragment of the 35S transcript (complement of the nucleotide sequence of SEQ ID No 12 from the nucleotide at position 1767 to the nucleotide at position 1991).

[085] GL4::ISP2A comprising the following DNA fragments:

the GL4 promoter region (SEQ ID No 2);

a DNA fragment encoding the isp2A protein of *Brevibacillus laterosporus* (complement of the nucleotide sequence of SEQ ID 12 from the nucleotide at position 6001 to the nucleotide at position 7228); and

the 3' end fragment of the 35S transcript (complement of the nucleotide sequence of SEQ ID No 12 from the nucleotide at position 5765 to the nucleotide at position 5989).

[086] GL5::ISP1A comprising the following DNA fragments:

the GL5 promoter region (complement of the nucleotide sequence of SEQ ID No 13 from the nucleotide at position 4518 to the nucleotide at position 5822);

a DNA fragment encoding the ispA1 protein of *Brevibacillus laterosporus* (complement of the nucleotide sequence of SEQ ID 13 from the nucleotide at position 2003 to the nucleotide at position 4511); and

the 3' end fragment of the 35S transcript (complement of the nucleotide sequence of SEQ ID No 13 from the nucleotide at position 1767 to the nucleotide at position 1991).

[087] GL5::ISP2A comprising the following DNA fragments:

the GL5 promoter region (complement of the nucleotide sequence of SEQ ID No 13 from the nucleotide at position 8628 to the nucleotide at position 7324);

a DNA fragment encoding the isp2A protein of *Brevibacillus laterosporus* (complement of the nucleotide sequence of SEQ ID 12 from the nucleotide at position 6090 to the nucleotide at position 7317); and

the 3' end fragment of the 35S transcript (complement of the nucleotide

sequence of SEQ ID No 12 from the nucleotide at position 5765 to the nucleotide at position 5989).

[088] *Example 4. Expression analysis of chimeric genes comprising the GL4 and GL5 promoters in stably transformed corn plants.*

[089] The chimeric genes described in Example 3 were introduced in a T-DNA vector along with a chimeric bar gene (such as described in US patent 5,561,236) to yield pTWV011 (GL4 promoter constructs) and pTWV018 (GL5 promoter constructs). These T-DNA vectors were introduced in *Agrobacterium tumefaciens* containing the helper Ti-plasmid pGV4000 or pEHA101.

[090] Corn plants stably transformed with the mentioned chimeric genes were obtained using the *Agrobacterium* -mediated transformation technique described in US 6,140,553.

[091] Corn plants stably transformed with the mentioned chimeric genes may also be obtained using direct DNA delivery to corn protoplast as described in US 5,767,367.

[092] RNA was isolated from root, leaf and stem tissue of these corn plants (grown either *in vitro* or *in vivo*) and submitted to Northern analysis using a ISP1A specific probe. The individual results are represented in Table 3.

[093] In summary, after correction for loading by hybridization with ribosomal RNA probe, the GL5 promoter region on average initiated 11 times more transcription in roots than in leaves and 19 times more in roots than in stems.

[094] The GL4 promoter region on average initiates 2 times more transcription in roots than in leaves and more than 10 times more in roots than in stems (although individual transformants may exhibit a more pronounced corn root selective expression pattern).

[095] Table 2. Summary of GL4/GL5 transcription data

		mean	SD		
		n=3			
Gl4 promoter	root	1.25	1.18	root/leaf	2
	leaf	0.61	0.73	root/stem	>10
	stem	<0.06			
Gl5 promoter	root	1.32	0.24	root/leaf	11
	leaf	0.12	0.05	root/stem	19
	stem	0.07	0.03		

[096] Table 3. Summary of Northern analysis data

	Corn line			lsp1a mRNA (pg /µg tot. RNA)		
pTWV018 GL5 promoter						
1	G2ZM3598-004	root	*in vitro*	**1.07**		
2		root		**1.57**		
3		leaf	*in vivo*	**0.15**	root/leaf	10.2
4		stem		**0.06**	root/stem	26.2
5	G2ZM3595-014	root	*in vitro*	**2.17**		
6		root		**1.10**		
7		leaf	*in vivo*	**0.15**	root/leaf	7.3
8		stem		**0.10**	root/stem	11
9	G2ZM3595-018	root	*in vitro*	**1.39**		
10		root		**1.28**		
11		leaf	*in vivo*	**0.07**	root/leaf	18.3
12		stem		**0.04**	root/stem	32
13	G2ZM3596-016	root	*in vitro*	**0.73**		
14		root		**<0.06**		
15		leaf	*in vivo*	**<0.06**	root/leaf	nd
16		stem		**<0.06**	root/stem	nd

pTWV011 GL4 promoter			isp1a mRNA (pg /µg tot. RNA)		
1	G2ZM3592-029	root *in vitro*	**0.53**		
2		root	**0.72**		
3		leaf *in vivo*	**0.08**	root/leaf	9
4		stem	**<0.06**	root/stem	>12
5	G2ZM3592-030	root *in vitro*	**1.45**		
6		root	**2.60**		
7		leaf *in vivo*	**1.45**	root/leaf	1.8
8		stem	**<0.06**	root/stem	>43
9	G2ZM3593-002	root *in vitro*	**0.34**		
10		root	**0.43**		
11		leaf *in vivo*	**0.31**	root/leaf	1.4
12		stem	**<0.06**	root/stem	>7.2

[097] *Example 5. Expression analysis of chimeric genes comprising the GL4 and GL5 promoters in progeny of stably transformed corn plants.*

[098] Nine transgenic T0 lines of each of the GL4 and GL5 promoter containing corn plants of Example 4 were crossed with untransformed B73, and the T1 plants were analyzed by Southern blot, Northern blot and by ELISA assay for the presence of ISP1 mRNA or protein in various plant parts.

[099] Southern analysis was performed at the V4 stage to determine the copy number of the transgenes. All analyzed events were single copy events except two lines that contained 2 copies of the transgene.

[100] Northern analysis was performed on RNA derived from root, leaf and stem material obtained at the V11-V13 stage as in Example 4. Transcript levels were quantified using image quant analysis. A correction for loading differences was performed using a ribosomal probe.

[101] For the plants with the GL4 promoter containing transgene, isp1 mRNA was estimated between 0.17 to 0.74 pg/µg total RNA. The average isp1a mRNA level in roots (n=9) was 0.42 pg/µg total RNA (SE = 0.18). The average ratio (n=9) of isp1a mRNA in root versus leaf is >6.3. The average ratio (n=8) of isp1a mRNA in root versus stem is >7.1. No expression was seen in stem, except for one sample where the ratio root/stem was 1.9.

[102] For the plants with the GL5 promoter containing transgene, isp1 mRNA was estimated between 0.95 to 2.55 pg/µg total RNA. The average isp1a mRNA level in roots (n=9) was 1.57 pg/µg total RNA (SE = 0.53). The average ratio (n=9) of isp1a mRNA in root versus leaf is >17.6. The average ratio (n=8) of isp1a mRNA in root versus stem is >26.6. No expression was observed in stem.

[103] Root, leaf and stem material, harvested at the V8 stage, was analyzed at the protein level for the presence of ISP1a protein by ELISA. Two plants per event were analyzed. As a negative control, root leaf and stem for wtB73 was checked for ISP1A protein. No ISP1A protein was detected in the control experiments.

[104] For all events no ISP1A protein expression was detected in leaves or stem. The mean value of levels of ISP1A protein detected in roots were:

0.07 pg/ml corresponding to about 0.024% of total protein level (n=18) for roots of plants containing the GL4 promoter driven transgene.

0.12 pg/ml corresponding to about 0.041% of total protein level (n=18) for roots of plants containing the GL5 promoter driven transgene.

[105] Root, leaf, stem and pollen material, harvested at the flowering stage, was analyzed at the protein level for the presence of ISP1a protein by ELISA. One plant per event was analyzed. As a negative control, root, leaf, stem and pollen material for wtB73 was checked for ISP1A protein. No ISP1A protein was detected in the control experiments.

[106] For all events, no ISP1A protein expression was detected in pollen.

[107] Mean ISP1A Protein level detected in root, leaf and stem of plants containing the GL4 promoter driven transgene:

mean value (n=9)

root: 0.286µg isp1a/ml ~ 0.116% isp1a of tot. protein level

leaf: 0.018µg isp1a/ml ~ 0.0022% isp1a of tot. protein level (6% of root level)

stem: 0.020µg isp1a/ml ~ 0.0043% isp1a of tot. protein level (7% of root level)

[108] Mean ISP1A Protein level detected in root, leaf and stem of plants containing the GL4 promoter driven transgene

mean value (n=9):

root: 0.265µg isp1A/ml ~ 0.142% isp1a of tot. protein level

leaf: 0.013µg isp1A/ml ~ 0.0015% isp1a of tot. protein level (5% of root level)

stem: 0.024µg isp1A/ml ~ 0.0058% isp1a of tot. protein level (9% of root level)

[109] At seed setting, ISP1A protein level was determined in kernels of the transgenic plants. No ISP1A protein could be detected in seed of the transgenic plants, nor in the wt B73 control.